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Linkage of *Eco*RI Dissociation from its Specific DNA Recognition Site to Water Activity, Salt Concentration, and pH: Separating their Roles in Specific and Non-specific Binding

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We have measured the dependencies of both the dissociation rate of specifically bound *Eco*RI endonuclease and the ratio of non-specific and specific association constants on water activity, salt concentration, and pH in order to distinguish the contributions of these solution components to specific and non-specific binding. For proteins such as *Eco*RI that locate their specific recognition site efficiently by diffusing along non-specific DNA, the specific site dissociation rate can be separated into two steps: an equilibrium between non-specific and specific binding of the enzyme to DNA, and the dissociation of non-specifically bound protein. We demonstrated previously that the osmotic dependence of the dissociation rate is dominated by the equilibrium between specific and non-specific binding that is independent of the osmolyte nature. The remaining osmotic sensitivity linked to the dissociation of non-specifically bound protein depends significantly on the particular osmolyte used, indicating a change in solute-accessible surface area. In contrast, the dissociation of non-specifically bound enzyme accounts for almost all the pH and salt-dependencies. We observed virtually no pH-dependence of the equilibrium between specific and non-specific binding measured by the competition assay. The observed weak salt-sensitivity of the ratio of specific and non-specific association constants is consistent with an osmotic, rather than electrostatic, action. The seeming lack of a dependence on viscosity suggests the rate-limiting step in dissociation of non-specifically bound protein is a discrete conformational change rather than a general diffusion of the protein away from the DNA.

Keywords: protein-DNA interactions; osmotic stress; *Eco*RI restriction endonuclease; dissociation rate; bound water

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Introduction

Despite the explosion in the number of DNA-protein structures reported in the literature, the connection among molecular interactions, thermodynamics, and structure that defines the strength and specificity of sequence-specific binding is still not well understood. While it is generally conceded that hydration water likely plays an important energetic role in specific sequence recognition,[1–4] there are few methods that can probe its contribution. We have used an approach we term the osmotic stress method to measure both differences in water sequestered by DNA protein complexes[5] and, in one instance, a work to remove water from a non-cognate DNA-protein complex.[6] We have particularly focused on DNA complexes of the restriction endonuclease *Eco*RI as a model system for delineating the role of water in specific recognition. We measure a difference in the number of water molecules sequestered by specific and non-specific complexes of *Eco*RI from the dependence of the free energy difference between specific and non-specific binding on water chemical potential (osmotic pressure), in much the same way as a difference in bound ions or protonation is extracted from the sensitivity of a free energy difference to salt activity or pH, respectively. Water activity can be varied by adding a solute (osmolyte) that does

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not bind directly to the DNA or protein. The method measures differences in the water associated with complexes that excludes solute. Reactions that result in a change in solute-exposed surface area typically show differences in ''bound'' water that depend on the identity (size and chemical nature) of the osmolyte probing the surface, indicating an exclusion of solutes due to ''crowding''[7,8] or ''preferential hydration''.[9,10] Reactions that entail changes in cavities, pockets, or crevices that sterically exclude solutes typically are not sensitive to the nature of the solute. We found[5] that the non-specific *Eco*RI complex sequesters about 110 more water molecules at 20 °C than the specific complex with the recognition sequence GAATTC. Even a complex with a sequence that differs by only one base-pair from the specific site, the ''star'' sequence TAATTC, sequesters about the same number of water molecules as the totally non-specific complex at low osmotic pressures.[6] The insensitivity of the measured number of water molecules to the solute identity implies that these water molecules are sterically sequestered from solutes. We postulated that this water might occupy the DNA-protein interface of non-specific complexes; 110 water molecules would correspond to about 1.5 layers at this interface.

The observed dependence of the equilibrium-binding constant on water activity should be reflected in the dissociation rate constant of *Eco*RI from its recognition site. Indeed, we recently showed[11] that the lifetime of the specific *Eco*RI-DNA complex is dramatically longer in the presence of betaine or sucrose; increasing the betaine concentration from 0 to 3 m, for example, slows the dissociation of the complex by a factor of about 260.

Although most thermodynamic work has focused on equilibrium constants for DNA-protein complex formation, studies of dissociation rates (k_d) of DNA-protein complexes are also extensive. Dissociation kinetics are important in themselves, for understanding reaction rates of nucleases, ligases, polymerases, and repair enzymes, for example, and because the binding of regulatory proteins to their target DNA sequences within a cell may be controlled kinetically and not an equilibrium reaction. Often the equilibrium constant and dissociation rate show similar dependences on salt concentration and pH.[12] Differences, however, can lead to a better formulation of the detailed binding scheme. In particular, the dissociation of many DNA-protein complexes, including the specific *Eco*RI-DNA complex, occurs in two steps.[12,13] There is transition between specific and non-specific binding of the protein to the DNA. The non-specifically bound protein diffuses linearly along the DNA and either returns to the specific binding site or dissociates from the DNA. Differences in the sensitivities of relative equilibrium constants for specific and non-specific binding and dissociation rates on solution conditions (salt concentration, pH, water activity, etc.) can distinguish between factors that are in common and factors that are different for specific and non-specific binding. We have, therefore, now measured the sensitivity of the dissociation rate to water activity, extending our previous measurements of the osmotic dependence of *Eco*RI relative binding constants. In addition, we have measured the salt and pH-sensitivities of both the dissociation rate and the ratio of specific and non-specific binding constants.

We find that the dependence of the dissociation rate on water activity is dominated by the difference in 110 sequestered water molecules between the specific and non-specific binding modes of the protein at 20 °C. The remaining sensitivity of k_d to water activity is due to dissociation of the non-specifically bound protein from the DNA. The number of water molecules linked to this step is clearly dependent on the identity of the particular solute probing the reaction, varying between about −10 and +40 water molecules for the set of solutes used (neglecting the possible effect of viscosity changes). This behavior is expected for a reaction that involves a change in solute-accessible surface area.

The salt-dependence of the overall off rate is dominated by the dissociation of the non-specifically bound protein from the DNA, as concluded by others.[12] The residual salt-sensitivity associated with the equilibrium between specific and non-specific binding of the enzyme measured by a competitive binding assay is consistent with an osmotic rather than electrostatic action of salt. The number of water molecules coupled with the rate of dissociation is insensitive to salt concentration between about 75 mM and 130 mM NaCl. Water and salt activities act as independent thermodynamic parameters. It has been known for some time that at lower salt concentrations the specific site equilibrium binding constant,[14] and the K_m and k_{cat} parameters characterizing the enzymatic cleavage reaction[15] are all markedly less sensitive to ionic strength. We find that this abrupt change in salt-sensitivity is accompanied by an abrupt decrease in the number of water molecules linked to dissociation.

A strong dependence of the dissociation rate on pH is observed. Since we observe virtually no pH-dependence of the equilibrium between specific and non-specific binding measured by our competition assay, pH affects only the dissociation of the non-specifically bound complex from the DNA. The pH-dependence can be fit adequately by assuming two titrating groups with identical pK values. Since the number of salt ions linked to dissociation also increases by about two between pH 8.5 and 6.5, the titrating groups are likely in close contact with the DNA backbone. The number of water molecules coupled to the rate of dissociation is insensitive to pH. Water activity and pH are acting independently to affect binding and dissociation.

These results clearly demonstrate the importance of water compared with salt and pH in dis-

tinguishing specific and non-specific *Eco*RI binding. The results suggests also that osmotic stress might be a convenient way to increase the stability and lifetime of weak complexes for separation, chemical modification, or measurement of physical properties.

Results

Dissociation kinetics

Figure 1 shows the results of a typical gel shift assay used for quantifying dissociation kinetics. *Eco*RI is initially incubated with a 240 bp DNA fragment containing the specific recognition sequence. A 360 bp fragment derived from the same plasmid and containing the same recognition site and surrounding sequences is added to the reaction and allowed to incubate further before electrophoresis. The gel shows the loss of 240 bp complex and the concomitant gain of 360 bp complex with increasing time. At equilibrium, the fraction of each fragment with bound protein is the same.

Figure 2 shows semilog plots for the dissociation of *Eco*RI from its recognition sequence at 20 °C, 90 mM NaCl, pH 7.0, and with 0 or 0.6 m α-methyl glucoside. We plot a normalized difference in fragment fractions with bound protein as outlined in Methods and Materials (equation 12). This procedure has the advantage of correcting each point for small errors in the concentrations of DNA and protein. Additionally, since the total bound protein is measured, we can ensure that the *Eco*RI remains active throughout the time-course of the experiment (on a time-scale measured in hours).

As seen in Figure 2, the dissociation of *Eco*RI is well described by a single exponential and the rate of dissociation is sensitive to osmolyte concentration. Both kinetic curves show an apparent five to ten minute lag time that may reflect the further exchange of protein in the electrophoresis well. Increasing the 360 bp fragment concentration fourfold has <10 % effect on the dissociation rate (data not shown), indicating that an exchange of *Eco*RI between fragments does not occur *via* a second-order transfer reaction.

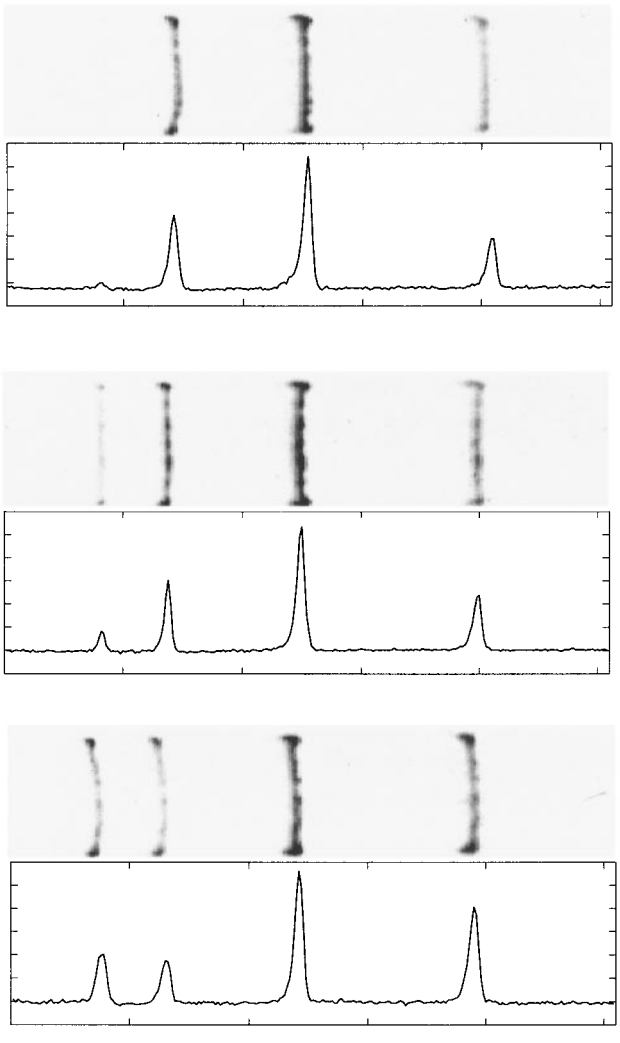

Figure 1. Typical gel lanes and their corresponding density profiles illustrating the measurement of specific site dissociation kinetics. *Eco*RI (~1 nM) was initially incubated with a 240 bp DNA fragment (~ 2 nM in sites) containing the specific recognition GAATTC sequence. A 360 bp fragment also containing the specific recognition site was then added to the mixture and allowed to react for the indicated times before electrophoresis. The DNA was stained afterwards with the dye SYBR Green I and a digital fluorescent image acquired. Dissociation is seen as the loss of *Eco*RI-240 bp complex and appearance of the *Eco*RI-360 bp complex. At equilibrium, the fractions of complex of each fragment are equal. Areas under the peaks were used to calculate fractions of bound and free fragments. The particular conditions used in this experiment were 90 mM NaCl, pH 7.0 (20 mM imidazole), and 20 °C with no added osmolyte. Under these conditions, enzyme binding is essentially stoichiometric.

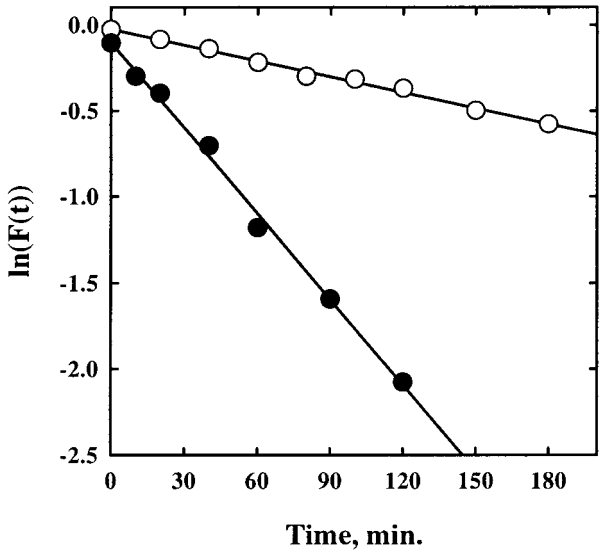

Figure 2. Specific site dissociation is exponential and strongly dependent on osmolyte concentration. Dissociation is measured through the loss of initially formed *Eco*RI-240 bp DNA complex and gain of the 360 bp DNA complex as described briefly in Figure 1. Semilog plots of the difference between the fraction of 240 bp fragment with bound protein and the fraction of 360 bp fragment present as complex normalized by the total protein (equation (12)) *versus* time are shown for two experimental conditions, with no added osmolyte (●) and in 0.6 m α-methyl glucoside (○). Both reactions mixtures also contained 90 mM NaCl, pH 7.0 (20 mM imidazole), and were incubated at 20 °C.

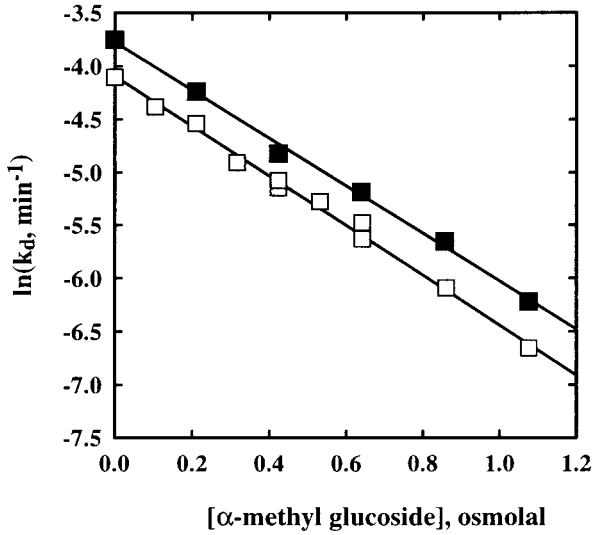

Figure 3. The dependence of $\ln(k_d)$ on osmolal concentration for two *Eco*RI sources: ■, *Eco*RI commercially obtained from New England Biolabs; □, highly purified *Eco*RI provided by Dr L. Jen-Jacobson. In addition to the added osmolyte, reactions were performed in 90 mM NaCl, at pH 7.0 (20 mM imidazole) and 20 °C. The linear dependence indicates that α-methyl glucoside is acting indirectly through water activity and that dissociation takes up water. The slopes of the two lines are virtually identical and correspond to 125(±10) water molecules.

Osmotic stress-dependence of k_d and K_{nsp-sp}

The same linkage relations that are commonly used to relate changes in equilibrium constants to changes in salt concentration, pH, temperature, or water activity are applicable to rate constants.[12] Just as the dependence of an equilibrium binding constant on salt or water activity, for example, gives the difference in the number of ions or water molecules associated with products and reactants, the sensitivity of a rate constant to salt or water activities is determined from the difference in the number of ions or water molecules associated with the initial state and the transition state of the rate-limiting kinetic step. Figure 3 shows the dependence of log (k_d) on the water chemical potential of the solution (expressed as an osmolal concentration of α-methyl glucoside) for *Eco*RI obtained commercially from New England Biolabs that we have used previously[5,6,11] and for highly purified nuclease kindly provided by Dr Jen-Jacobson. Both enzyme preparations show linear plots over the range of 0- ~ 1.2 osm. We have generally concluded that linear plots of ln(k) or ln(K) *versus* osmolal concentration (including the no added solute limit) indicate an osmotic action. This interpretation is further strengthened if the slope of the line is relatively insensitive to the size and chemical nature of the osmolyte. We have found that the free energy difference between specific and non-specific binding of *Eco*RI does vary linearly with water chemical potential and that this effect is insensitive to the identity of the solute.[5] We will, therefore, analyze solute effects on dissociation rates here also as osmotic. The sensitivity of the dissociation rate on water activity, for example, is determined by the difference in the numbers of water molecules associated with the initial specific complex of the enzyme with DNA and the transition state of the rate-limiting kinetic step of dissociation. Neglecting for the moment the possible contribution from changes in bulk solution viscosity, a difference in the number of solute-excluding water molecules between the two states, $\Delta N_w^{\dagger}$, can be evaluated from:

$$\frac{d\ln(k_d)}{d(osm)} = -\frac{\Delta N_w^{\dagger}}{55.6} \qquad (1)$$

The dissociation rate of the highly purified enzyme (Figure 3) is about 30 % slower than that of the commercially obtained *Eco*RI. The slopes of the two plots are virtually identical, translating into a net uptake of an additional 125(±10) water molecules coupled to dissociation.

We have also confirmed that the equilibrium properties of the two enzyme preparations are closely similar. Figure 4 compares the dependence of

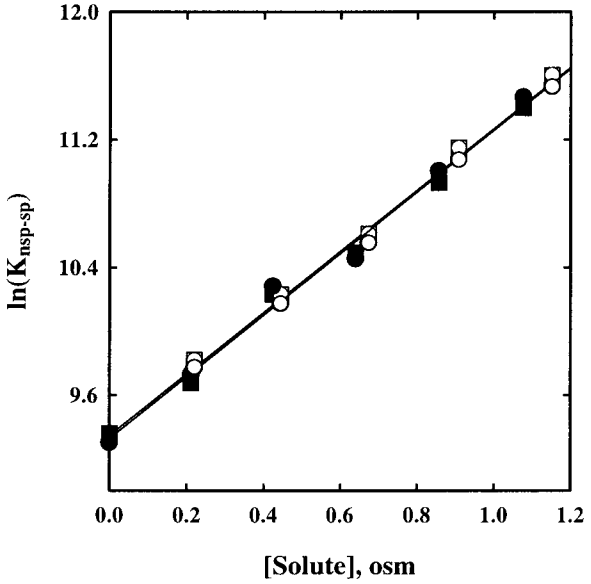

Figure 4. No difference between *Eco*RI purchased from New England Biolabs that we used previously and highly purified *Eco*RI kindly provided by Dr L. Jen-Jacobson is seen in the osmotic stress-dependence of the equibrium between specific and non-specific binding. The ratios of specific and non-specific *Eco*RI binding constants were determined from a direct competition assay using a 240 bp DNA fragment containing *Eco*RI specific recognition site and the non-specific oligonucleotide competitor $(dI\text{-}dC)_{12}$. Mixtures of *Eco*RI (~1 nM), the 240 bp DNA fragment (~2 nM in recognition sites), and the oligonucleotide competitor (~0-20 μM of oligonucleotide) were incubated for 90 minutes in 100 mM NaCl, pH 8.0 (20 mM imidazole), and at 20 °C in the absence of Mg^{2+}. The squares and circles show the data for *Eco*RI purchased from New England Biolabs and provided by Dr L. Jen-Jacobson, respectively. Two osmolytes were used, betaine glycine (□,○) and α-methyl glucoside (■,●). The slope of the plot indicates that the non-specific complexes of *Eco*RI with $(dI\text{-}dC)_{12}$ sequesters some 110 (±10) more water molecules than the specific site complex.

the specific-non-specific binding equilibrium for *Eco*RI on water chemical potential (solute osmolal concentration) both for the enzyme purchased from New England Biolabs and for the enzyme provided by Dr Jen-Jacobson. As described in Materials and Methods, the ratio of specific and non-specific complex association constants (K_{nsp-sp}) was determined from a direct competition assay using the oligonucleotide $(dI\text{-}dC)_{12}$ as the non-specific competitor. Both betaine glycine and α-methyl glucoside were used to vary water activity. No difference in K_{nsp-sp} or its osmotic dependence is observed between the two proteins. The difference in sequestered water between specific and non-specific complexes, $\Delta N_{w,nsp-sp}$, extracted from the slope is −105(±8) water molecules, in agreement with our previous results[5] using seven different osmolytes. The 125 water molecules seen linked to dissociation, therefore, corresponds to about 15-20 more water molecules than seen for the equilibrium between specific and non-specific *Eco*RI binding for these two osmolytes.

Figure 5 shows plots of $\ln(k_d)$ *versus* osm for the highly purified enzyme in 90 mM NaCl and pH 7.0, and at 20 °C for a wide variety of solutes. All of the solutes are closely similar in their ability to slow dissociation. There is, however, more solute-specific variation than we observed previously for K_{nsp-sp}. Stachyose, α-methyl glucoside, t-butanol, and betaine glycine all give slopes that translate into 120-130 water molecules (within about 10 % error). Triethylene glycol (155(±8) water molecules), DMSO (145(±8) water molecules), and trimethylamine N-oxide (TMAO) (155(±4) water molecules) give slopes ~20 % higher. The slope with sucrose is somewhat smaller than the others corresponding to only 100(±6) water molecules. It should be noted that the osmotic sensitivity of the free energy difference between specific and non-specific equilibrium binding for sucrose was within experimental error of the results for betaine glycine, α-methyl glucoside, stachyose, and triethylene glycol.[5]

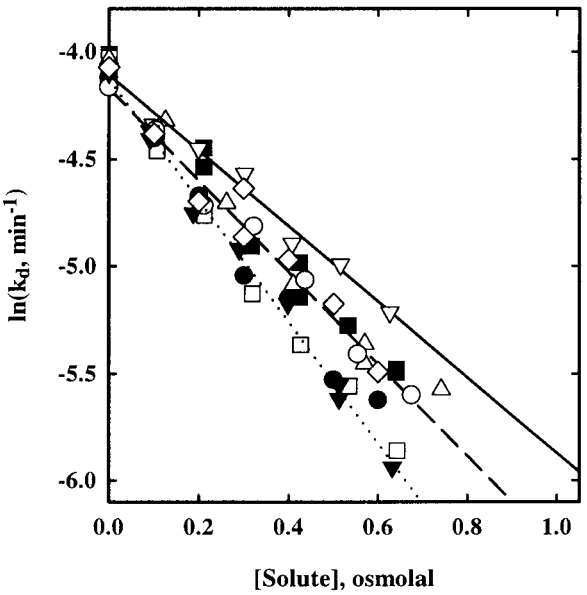

Figure 5. The dependence of $\ln(k_d)$ on solute osmolal concentration for the highly purified *Eco*RI for a wide variety of osmolytes: ▽, sucrose; ■, α-methyl glucoside; ◇, *t*-butanol; △, stachyose; ○, betaine; ▼, TMAO; ●, DMSO; □, triethylene glycol. The slopes of the lines translate into 120(±8) water molecules for stachyose, 125(±6) for α-methyl glucoside, 120(±10) for t-butanol, and 120(±6) for betaine glycine. Triethylene glycol (155(±8) water molecules), DMSO (145(±8) water molecules) and TMAO (155(±4) water molecules) give slopes ~20 % higher. Sucrose gives the smallest slope (100(±6) water molecules). The average errors for each $\ln(k_d)$ value did not exceed 10 %. In addition to the added osmolyte, all reactions were performed in 90 mM NaCl, at pH 7.0 (20 mM imidazole) and 20 °C.

Salt-dependence of k_d and K_{nsp-sp}

Figure 6(a) shows $\log(k_d)$ *versus* $\log(C_{salt})$ at several different water activities at 20 °C and pH 7.5. Above about 60 mM NaCl, the best fitting lines are parallel for the three concentrations of osmolyte. Between 60 and 130 mM NaCl, the slopes correspond to the binding of 5.8(±0.5) ions linked to *Eco*RI dissociation. This compares with $\Delta N^{\dagger}_{salt} = 5.9$ ions reported previously by Jen-Jacobson and coworkers[16,17] for the dissociation rate of *Eco*RI at pH 7.4. The salt-dependence of the equilibrium between free and specifically bound *Eco*RI has been reported as $\Delta N_{salt} \sim 8$ ions[14] and, more recently, as ~10-11 ions.[4] Below about 60 mM NaCl, the salt-dependence actually inverts, as has been seen for equilibrium measurements.[14] The steady-state enzymatic parameters K_m and k_{cat} lose their salt-sensitivity in about the same range of salt concentration.[15] It has been suggested[14] that protein aggregation is responsible for this behavior. We see, however, no loss of protein during the course of the experiment. An inverted salt-dependence would be consistent with the formation of an intermediate *Eco*RI complex containing two DNA fragments. The dissociation rate, however, changes by less than 10 % with a fourfold increase in the 360 bp fragment concentration.

Figure 6(b) shows the osmotic sensitivity of the dissociation rate at pH 7.5 and 20 °C for several salt concentrations, using both betaine glycine and α-methyl glucoside to vary water activity. At 75 and 90 mM NaCl, the slopes are reasonably constant within experimental error, with $\Delta N^{\dagger}_{w} = 125(\pm 10)$ water molecules. The slope at 125 mM NaCl is somewhat smaller, corresponding to 110(±9) water molecules. This may reflect a small change in the structure of the non-specific complex or a small coupling of ion and water binding sensitivity to activities at these high osmotic pressures.

Much larger changes in $\Delta N^{\dagger}_{w}$ are seen at salt concentrations below 60 mM, in the region that also shows the inverted salt-sensitivity. The slopes translate to $\Delta N^{\dagger}_{w} = 65(\pm 5)$ water molecules and 30(±6) water molecules for 50 and 40 mM NaCl, respectively. Both the salt and osmotic sensitivities indicate a fundamental change in the character of the dissociation reaction of enzyme from DNA below about 60 mM NaCl for these experimental conditions.

The effect of salt on the equilibrium between specific and non-specific binding of *Eco*RI at 20 °C and pH 8.0 is shown in Figure 7. In contrast to the about 20-fold increase in dissociation rate expected between 90 and 160 mM NaCl, K_{nsp-sp} increases by only about 50 %. Figure 7 shows the data analyzed as an indirect, osmotic action of salt rather than an electrostatic effect. The observed slope corresponds to an extra 150(±40) water molecules associated with the non-specific complex compared with specific one. This number is only somewhat higher than the difference of ~110 water molecules

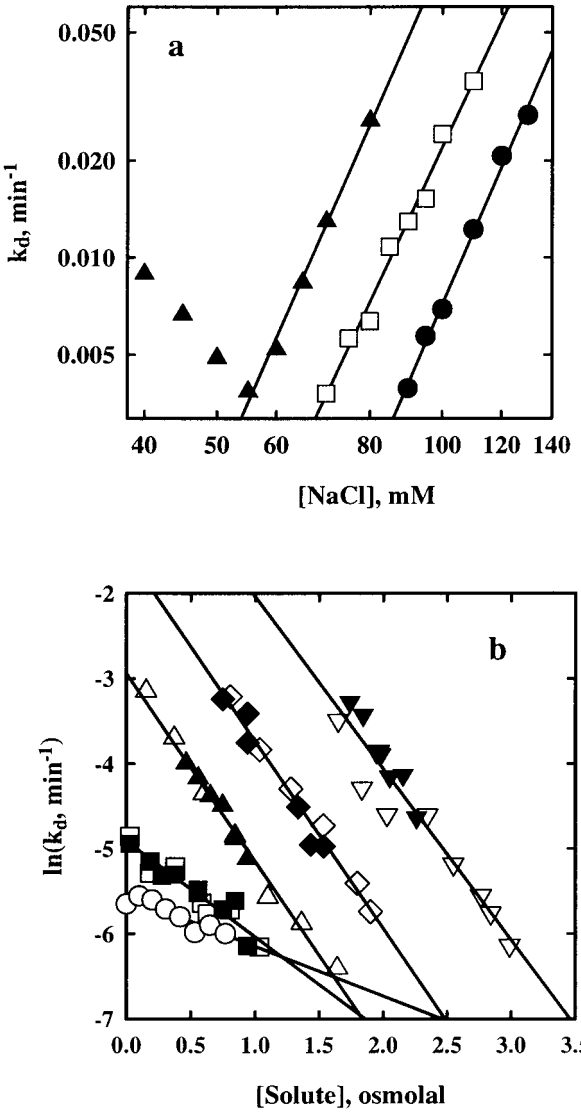

Figure 6. (a) The dependence of k_d on NaCl concentration for different water activities (osmolyte concentrations): ▲, 0.5 m; □, 1.2 m; and ●, 1.6 m betaine glycine. Between 60-130 mM NaCl, the slopes of these log-log plots are the same for the three osmolyte concentrations and correspond to the binding of 5.8(±0.6) ions linked to *Eco*RI dissociation. Below about 60 mM NaCl, the salt-dependence actually inverts. All reactions were performed at pH 7.5 (20 mM imidazole) and 20 °C. (b) The dependence of $\ln(k_d)$ on osmolal concentrations of betaine glycine and α-methyl glucoside for the highly purified *Eco*RI for several NaCl concentrations. 125 mM NaCl: ▽, betaine; ▼, α-methyl glucoside; 90 mM NaCl: ◇, betaine; ◆, α-methyl glucoside: 75 mM NaCl: △, betaine; ▲, α-methyl glucoside; 50 mM NaCl: □, betaine; ■, α-methyl glucoside: 40 mM NaCl: ○, betaine. At 75 and 90 mM NaCl, the slopes are same within experimental error, with $\Delta N^{\dagger}_{w} = 125(\pm 10)$ water molecules. The slope at 125 mM NaCl is somewhat smaller, corresponding to 115(±9) water molecules. All reactions were performed at pH 7.5 (20 mM imidazole) and 20 °C.

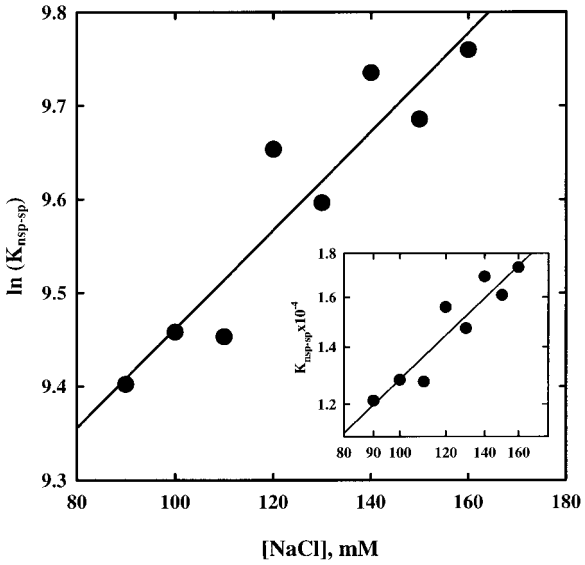

Figure 7. The dependence of the ratio of *Eco*RI specific and non-specific binding constants, K_{nsp-sp}, on NaCl concentration. The relative binding constant, K_{nsp-sp}, was determined from the loss of specific site binding to a 240 bp DNA fragment as the concentration of non-specific competitor oligonucleotide, $(dI\text{-}dC)_{12}$, is increased. In contrast to the ~20-fold increase in dissociation rate expected between 90 and 160 mM NaCl, K_{nsp-sp} increases by only about 50%. The data are shown plotted as an osmotic effect, $\ln(K_{nsp-sp})$ *versus* [NaCl] (in this range, salt osmolal concentration is 1.86 [NaCl]). The slope corresponds to a release of 150(±40) water molecules in going from the non-specific to specific *Eco*RI complex. All the neutral solutes examined previously showed a difference, $\Delta N_{w,nsp-sp}$, of −110 water molecules. The Figure inset shows the data plotted as an ion-binding effect, a log-log plot of K_{nsp-sp} *versus* [NaCl]. Analysed in this way, the slope corresponds to a release of 0.6(±0.1) ion going from the specific to the non-specific complex. All reactions were performed at pH 8.0 (20 mM imidazole) and 20 °C and with no added osmolyte.

between specific and non-specific *Eco*RI complexes we found previously using neutral solutes. If salt is excluded from the same water-filled space as the neutral solutes, then this osmotic contribution from salt is required. Alternatively, the inset in Figure 7 shows the data analyzed as a change in direct salt binding, i.e. as a log-log plot of K_{nsp-sp} *versus* C_{salt}, the observed slope corresponds to about 0.6 fewer ion associated with the non-specific complex than with the specific one. The data are not sufficiently precise over this range of salt concentrations to allow an unambiguous dissection of the effect of salt on the equilibrium between specific and non-specific binding.

pH-dependence of k_d and K_{nsp-sp}

Figure 8(a) shows the dependence of log (k_d) on pH for two buffers (Tris and imidazole) and four osmotic stresses. As the pH increases, the rate of dissociation increases. At the same osmotic stress, salt concentration, and pH, the rate of dissociation with Tris is about threefold faster than with imidazole. An effect of buffer identity on DNA-protein complex stability has been seen previously.[18] It may result from pK_a-dependent contributions of the buffer to the ionic strength of the solution.

The factor shift of dissociation rate between any two curves in overlapping pH regions is constant and can be used to correct the data to the 0.25 m α-methyl glucoside curve. Figure 8(b) shows the rescaled dissociation rate titration curve from pH 6.2 to 9.2. Over this region, k_d increases by about 40-fold. The plateau value of the rate is not as well defined in the higher pH region as in the lower pH region. This may indicate that additional protein groups are beginning to titrate in the higher pH range. The curve is too steep to be well fit by a model assuming a single titrating group with different dissociation rates for the protonated and unprotonated forms (the continuous line in Figure 8(b)). A single titrating group is, of course, inconsistent with the dimeric structure of the protein. The fit (the broken line) is somewhat better with two titrating groups that have identical pK values and activation energies that depend linearly on charge. The data are not sufficiently precise to justify further fitting parameters and more complicated models.

If the protein groups that are titrating over this pH range and affecting the dissociation rate are in close contact with DNA, particularly phosphate groups, then the pH-dependence will be accompanied by a change in the number of salt ions coupled with dissociation. Figure 9 shows the log-log dependence of k_d on C_{salt} at pH values of 6.5 (imidazole) and 8.5 (Tris). The data for each pH were collected using two osmolyte concentrations and overlapping salt concentrations (analogous to Figure 6(a)). The data have been extrapolated to zero osmolyte, but not corrected for the difference between imidazole and Tris buffers. At pH 8.5, $\Delta N^{\dagger}_{salt}$ is 5.3(±0.5) ions, while it is 7.2(±0.5) ions at pH 6.5. The $\Delta\Delta N^{\dagger}_{salt}$ ~2 suggests that two protein groups reasonably close to the sugar-phosphate backbone are titrating.

Figure 10 shows the osmotic pressure-dependence of $\ln(k_d)$ for α-methyl glucoside and betaine at pH values of 6.5, 7.5, and 8.5. The observed slopes translate into an apparent values $\Delta N^{\dagger}_{w}$ of 140(±15), 120(±6), and 110(±9) water molecules for pH 6.5, 7.5, and 8.5 respectively. As seen in Figure 6(b), there is again an approximate 15% decrease in $\Delta N^{\dagger}_{w}$ between the low and high osmotic stress regimes.

The equilibrium competition between specific and non-specific binding at pH 7.0 and 8.0 (120 mM NaCl, at 20 °C) is shown in Figure 11. The approximate 12-fold difference in dissociation rate between the two pH values (Figure 8) is not reflected in the relative binding constant, which changes by less than 10%. There is no significant

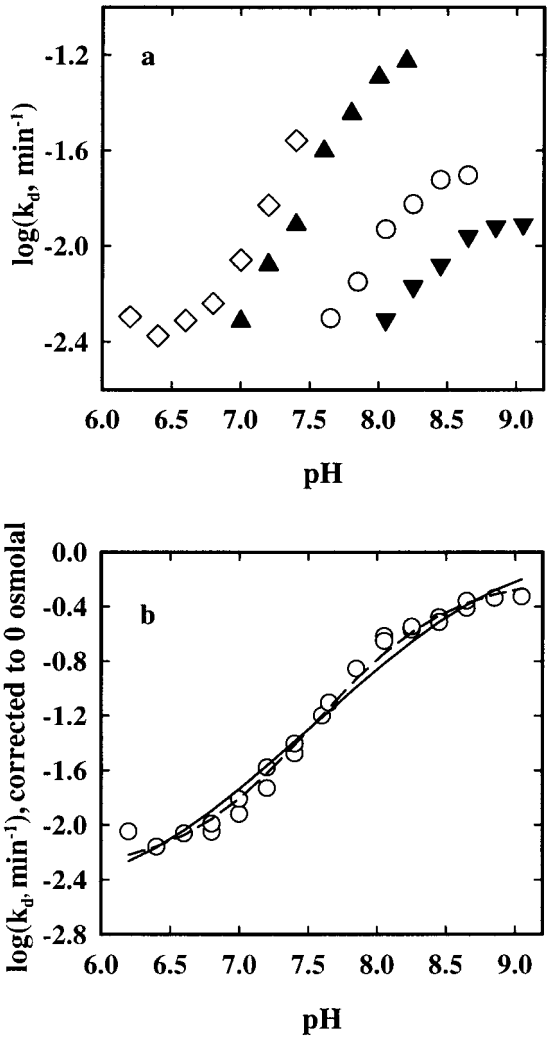

Figure 8. (a) The dependence of k_d on pH for two buffers (20 mM Tris and 20 mM imidazole) and four osmolyte concentrations: ◇, imidazole, 0.25 m α-methyl glucoside; ▲, imidazole, 1.0 m α-methyl glucoside; ○, imidazole, 1.5 m α-methyl glucoside; and ▼, Tris, 2.0 m α-methyl glucoside. At the same osmotic stress and pH, the rate of dissociation with Tris is about threefold faster than with imidazole. All reactions were performed in 90 mM NaCl and at 20 °C. (b) The constant factor shifts in rate amplitudes between the overlapping pH regions of the various curves in (a) were used to rescale the data to the imidazole, 0.25 m α-methyl glucoside curve. The continuous line shows the best fit assuming a single protein group is titrating and that the dissociation rate is k_d^0 and $k_d^0 k_d^*$ for the unprotonated and protonated complexes, respectively (equation (15) in Materials and Methods). The best fitting parameters are $k_d^0 = 1.0\ \text{min}^{-1}$, $k_d^* = 3 \times 10^{-3}$, and $pK_H = 8.8$ for the specific complex. The broken line shows the best fit for two independently titrating protein groups with identical pK values, also assuming that each protonation slows the dissociation by a constant factor (equation (16) in Methods and Materials). The best fitting parameters are: $k_d^0 = 0.64\ \text{min}^{-1}$, $k_d^* = 0.085$, and $pK_H = 8.1$.

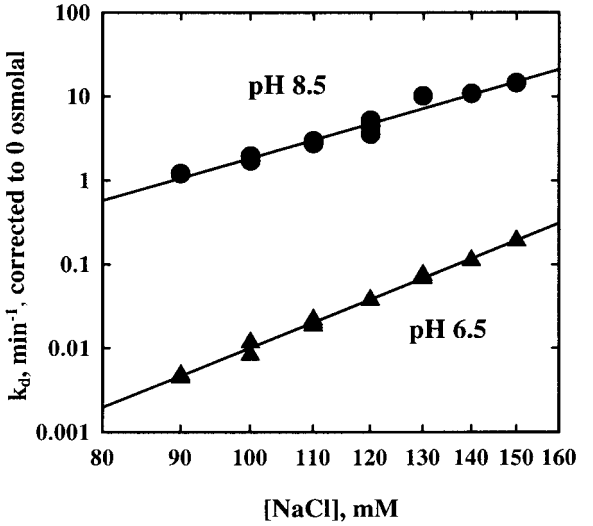

Figure 9. The dependence of k_d on salt concentration at pH 6.5 (▲) and 8.5 (●). As in Figure 6, the dependence of dissociation rate for each pH on salt concentration over the range of 90 - 150 mM NaCl was determined using two concentrations of the osmolyte α-methyl glucoside. For each pH, the log-log curves of k_d *versus* [NaCl] for the two osmolyte concentrations were parallel with each other. The data shown in the Figure have been extrapolated to zero osmolyte using $\Delta N_w^\dagger = 125$ water molecules, but not corrected for the difference between imidazole (pH 6.5) and Tris (pH 8.5) buffers. At pH 6.5, $\Delta N_{salt}^\dagger = 7.2(\pm 0.5)$ ions; at pH 8.5, $\Delta N_{salt}^\dagger = 5.3(\pm 0.5)$ ions.

difference in the pH-dependence of specific and non-specific binding of *Eco*RI.

Discussion

Many sequence-specific DNA-binding proteins, such as the restriction endonuclease *Eco*RI, initially bind non-specifically to DNA and diffuse linearly along the helix until either a specific recognition site is found or the protein dissociates from the DNA.[4,12,13,19–22] It has been estimated that in an ionic strength of 80 mM and at pH 7.6 non-specifically bound *Eco*RI, for example, will scan some 1300 base-pairs, on the average, before dissociating.[15,19] The rate of dissociation from a specific recognition site will therefore depend both on the reaction kinetics for the transition between the specific and non-specific binding of the enzyme to DNA in the vicinity of the specific site and on the subsequent dissociation rate of the non-specifically bound protein from DNA. The kinetic scheme shown in equation (10) in Methods and Materials can be modified to include a non-specifically bound intermediate state, [AP]*. In this state, the protein can slide along the DNA either to rebind to the specific recognition site or to dissociate from the DNA:

$$A + P \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} [AP]^* \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} AP \qquad (2)$$

.

Since the ratio of specific and non-specific association constants for *Eco*RI[5,6,23] is of the order of 10^4, the concentration of non-specifically bound intermediate can also be treated in a steady-state limit to give a dissociation rate:

$$k_d = \frac{k_2' k_4'}{(k_2' + k_3')(1 - f_\infty)} \qquad (3)$$

where f_∞ is the fraction of specifically bound DNA fragment at equilibrium.

The estimated value of $\sim 10^6$ for the ratio of sliding and dissociation probabilities for non-specifically bound *Eco*RI measured under similar experimental conditions[20] indicates that the protein will return to the specific site many times before finally dissociating from the DNA, i.e. $k_2' \ll k_3'$, or:

$$k_d = \frac{k_2'}{(1 - f_\infty)} \frac{k_4'}{k_3'} = \frac{k_2'}{(1 - f_\infty)} \frac{1}{K_{nsp-sp}} \qquad (4)$$

The ratio k_3'/k_4' is the equilibrium constant, K_{nsp-sp}, characterizing the ratio of specific and non-specific *Eco*RI binding to the DNA fragment.

In the other limit of very fast dissociation of non-specifically bound protein from the DNA compared with the rate of transition from specific to non-specific protein binding ($k_2' \gg k_3'$):

$$k_d = \frac{k_4'}{(1 - f_\infty)} \qquad (5)$$

The sensitivities of the dissociation rate for these two limiting expressions to salt, water, or proton activities are given by standard linkage relations:

$$\frac{d \ln(k_d)}{RT d \ln(a_s)} = \frac{d \ln(k_2')}{RT d \ln(a_s)} - \frac{d \ln(K_{nsp-sp})}{RT d \ln(a_s)} = \Delta N_s^\dagger = \Delta N_{s,2}^\dagger - \Delta N_{s,nsp-sp} \qquad (6)$$

or:

$$\frac{d \ln(k_d)}{RT d \ln(a_s)} = \frac{d \ln(k_4')}{RT d \ln(a_s)} = \Delta N_{s,4}^\dagger \qquad (7)$$

where a_s is the activity of salt, protons, or water and RT is thermal energy. For water, $RT\, d\ln(a_w) = -\, d(\text{osm}/55.6)$. For $k_2' \ll k_3'$, $\Delta N_{s,2}^\dagger$ and $\Delta N_{s,nsp-sp}$ are the differences in the numbers of associated water molecules, ions, or protons between the non-specifically bound complex and the transition state for protein dissociation and between *Eco*RI non-specifically and specifically bound complexes, respectively. For $k_3' \ll k_2'$, $\Delta N_{s,4}^\dagger$ is

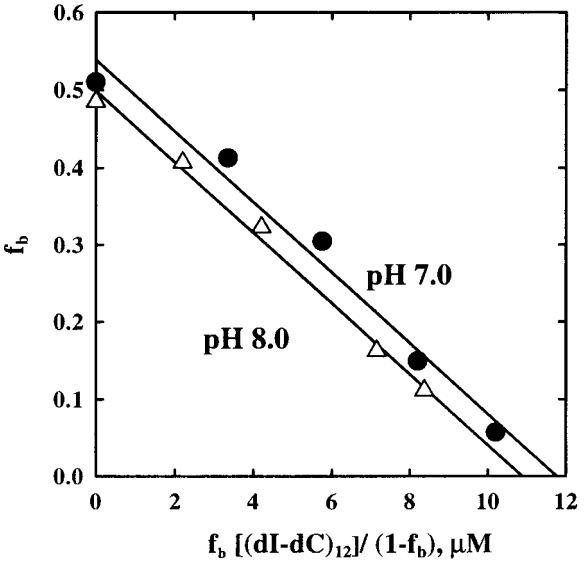

Figure 11. The equilibrium between specific and non-specific *Eco*RI binding is insensitive to pH between 7.0 (●) and 8.0 (△). The data are plotted as specified by equation (13) in Methods and Materials for the competitive binding assay. The loss of 240 bp fragment with specifically bound *Eco*RI, f_b, with increasing concentration of non-specific competitor oligonucleotide, $(dI\text{-}dC)_{12}$, was virtually identical at pH 7 and 8. The slopes, which are inversely proportional to K_{nsp-sp}, differ by less than 10 %. Reactions were performed in 20 mM imidazole, 120 mM NaCl, and at 20 °C.

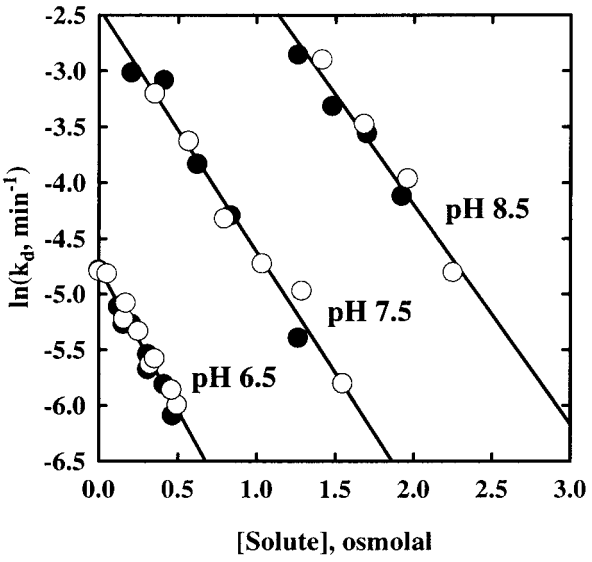

Figure 10. The dependence of $\ln(k_d)$ on water chemical potential (osmolal concentration) at pH 6.5, 7.5, and 8.5 for two osmolytes: ●, α-methyl glucoside, and ○, betaine glycine. The observed slopes translate into an apparent values for $\Delta N_w^\dagger$ of 140(±15), 120(±6), and 110(±9) water molecules for pH 6.5, 7.5, and 8.5, respectively. All reactions were performed in 90 mM NaCl and at 20 °C.

Table 1. The apparent number of water molecules coupled with the dissociation of specifically and, by inference, of non-specifically bound *Eco*RI, calculated from the osmolyte sensitivity of the rate constant and including a possible viscosity correction

Osmolyte	Molecular mass	$\Delta N^{\dagger}_{w}$ [a]	$\Delta N^{\dagger}_{w,2}$ [b]	$55.6\frac{d\ln(\eta)}{d(osm)}$ [c]	$\Delta N^{\dagger}_{w}-55.6\frac{d\ln(\eta)}{d(osm)}$ [d]	$\Delta N^{\dagger}_{w,2}-55.6\frac{d\ln(\eta)}{d(osm)}$ [e]
t-Butanol	74	120	10	20	100	−10
TMAO	75	155	45	10	145	35
DMSO	78	145	35	10	135	25
Betaine	117	120	10	10	110	0
Triethylene glycol	150	155	45	20	135	25
α-Methyl glucoside	194	125	15	25	100	−10
Sucrose	342	100	−10	40	60	−50
Stachyose	667	120	15	75	50	−60

Common conditions: 90 mM NaCl, pH 7.0 (20 mM imidazole).

[a] Total number of water molecules linked to the dissociation rate of specifically bound protein ($-55.6\ d(\ln(k_d))/d(osm)$), assuming no contribution from viscosity. Errors are given in the legend to Figure 5.

[b] The number of water molecules linked to the dissociation rate of non-specifically protein calculated using equation (6) and $\Delta N_{w,nsp-sp} = -110$ molecules. Again a possible viscosity contribution is neglected.

[c] The maximal contribution to the dissociation rate from viscosity expressed as an apparent number of water molecules, calculated from the dependence of viscosity on solute concentration (errors did not exceed 5%).

[d] The number of water molecules linked to the dissociation rate of specifically bound protein calculated using equation (9) assuming the maximal viscosity contribution.

[e] The number of water molecules linked to the dissociation rate of non-specifically bound protein calculated with $\Delta N_{w,nsp-sp} = -110$ molecules and assuming the maximal viscosity contribution.

the difference in the number of associated water molecules, ions, or protons between the *Eco*RI specifically bound complex and the transition state between the specific and non-specific states.

The large salt-dependence seen for the dissociation rate[16,17] (Figures 6(a) and 9) and for the equilibrium between free and specifically bound *Eco*RI,[4,14,16] but not for the equilibrium between specifically and non-specifically bound *Eco*RI (Figure 7) is consistent with the expectation that the dissociation of non-specifically bound protein from the DNA fragment is the slow step in the overall process, i.e. $k_2' \ll k_3'$. Otherwise, even though there is little or no difference in the numbers of associated ions, for example, between the specific and non-specific complexes, the transition state between the two would have to bind an additional five to seven ions in order to account for the salt-dependence in $\Delta N^{\dagger}_{salt,4}$, which seems unlikely.

Plots of $\ln(k_d)$ *versus* osmolal concentration in Figure 5 are linear for each solute (including the no osmolyte limit). Total $\Delta N^{\dagger}_{w}$ values extracted from the slopes for the eight solutes used are shown in Table 1. These changes in hydration show more solute variation than we observed previously for the equilibrium binding experiments,[5,6] but still much less than the factor differences of two to five expected for an osmotic effect based solely on exclusion of solutes from exposed surface areas.[9,10,24–27] We can estimate $\Delta N^{\dagger}_{w,2}$, the change in water associated with non-specifically bound protein dissociation using equation (6) and our previous measurement of $\Delta N_{w,nsp-sp} = -110$ water molecules that showed no osmolyte variation within experimental error. For the seven solutes other than sucrose, $\Delta N^{\dagger}_{w,2}$ is positive and varies from about ten to 45 extra water molecules associated with the k_2' transition state. This reaction step is likely characterized by a change in solute-accessible surface area. For sucrose, $\Delta N^{\dagger}_{w,2}$ is $-10(\pm 20)$ water molecules. This represents either no exclusion from or a small preferential inclusion of sucrose with the newly exposed surface area.

Viscosity

Simplified transition state theory expresses rate constants in terms of a frequency, ν, transmission coefficient, κ, and a free energy difference between the initial and transition states, $\Delta G^{\dagger}$, as:

$$\ln(k) = \ln(\nu\kappa) - \frac{\Delta G^{\dagger}}{RT} \qquad (8)$$

Solutes can affect rates by acting on $\Delta G\dagger$ through differential interactions with the initial and transition states (the basis of the linkage relations), and through ν by changing the solution viscosity. The effect of solution viscosity, η, on the rate of dissociation is often not straightforwardly apparent.[28–30] Kinetic steps that are characterized by motions limited by viscous dissipation of the bulk solution are expected to have rates that vary inversely with the solution viscosity. If, for example, diffusion of protein away from DNA was the slow step in the dissociation of non-specifically bound *Eco*RI, then solution viscosity would contribute maximally. In this limit, part of the change in the dissociation rate with solute concentration would be due to a change in viscosity. The apparent $\Delta N_{w,app,\eta}$ component that is due instead to a viscosity-dependence on solute osmolal concentration contributes to the overall change as:

$$\frac{d\ln(k_d)}{d(osm)} = \frac{d\ln(k_2')}{d(osm)} - \frac{d\ln(K_{nsp-sp})}{d(osm)} - \frac{d\ln(\eta)}{d(osm)} = \frac{\Delta N_{w,2}^{\dagger}}{55.6} + \frac{\Delta N_{w,nsp-sp}}{55.6} - \frac{\Delta N_{w,app,\eta}}{55.6} \quad (9)$$

Plots of ln(η) *versus* solute osmolal concentration are reasonably linear from 0 to 1.5 osm for all the solutes used here (data not shown). Table 1 gives the maximal contribution from solution viscosity changes expressed as an apparent number of water molecules for each solute and values for $\Delta N_{w,2}^{\dagger}$ calculated assuming viscosity contributes maximally. The number of water molecules linked to the equilibrium between specific and non-specific binding, $\Delta N_{w,nsp-sp}$, is, of course, independent of viscosity. For several of the solutes here (betaine, TMAO, and DMSO), even if the dissociation rate of protein from a non-specific complex was determined completely by the viscous drag of the solution, the viscosity correction to $\Delta N_{w}^{\dagger}$ is still less than 10 % of the measured value. The corrections are somewhat larger, but still not unreasonable (15-20 %), for triethylene glycol and t-butanol. The corrections are significantly larger for α-methyl glucoside, sucrose, and stachyose. With a viscosity correction, the total number of water molecules linked to the dissociation rate would be reduced to 100, 60, and 50 water molecules for α-methyl glucoside, sucrose, and stachyose, respectively. Since equilibrium constants are invariant with changing viscosities, all this change would be coupled to the dissociation of non-specifically bound protein. Values of $\Delta N_{w,2}^{\dagger}$ would now be −10, −50, and −60 water molecules for α-methyl glucoside, sucrose, and stachyose, respectively. In order for the protein dissociation rate to be limited by the bulk solution viscosity, these polyol solutes would have to be strongly preferentially included, and the inclusion must increase with increasing sugar size. Although it is possible that these solutes act very differently from the other osmolytes on the dissociation of protein, it is more likely that the slow step in dissociation is not limited directly by solution viscous drag and, therefore, is not a simple diffusion of protein from DNA. The rate-limiting motions leading to protein dissociation seem instead to be constrained by an ''internal friction'' of the complex rather than by the viscous dissipation of rigid body motion in the bulk solution. This suggests that the dissociation rate is limited by a discrete protein conformational change, perhaps of the N-terminal arms that are postulated to enfold the DNA.[16,31,32]

Salt and pH

In contrast to the osmotic-dependence, both salt and pH predominantly affect the dissociation of non-specifically bound protein, not the equilibrium between specific and non-specific binding. In the salt concentration range 60-130 mM NaCl, the numbers of ions and water molecules coupled with dissociation are virtually constant and independent (Figure 6(a) and (b)). The additional number of bound ions is the same for both α-methyl glucoside and betaine, even though α-methyl glucoside will lower the macroscopic dielectric constant of the bulk solution, while betaine will raise it. The number of extra water molecules bound accompanying dissociation does seem to decrease slightly (∼ 15 %) at higher salt concentrations, and the higher osmotic stresses necessary to slow the dissociation rate sufficiently to measure it. It is possible that there is a small, uncompensated dependence of ion binding and activities on solute concentration at the higher osmotic stresses. Alternatively, a similar small decrease in ΔN_{w} was measured for the equilibrium reaction at very high osmotic stresses.[6] It is possible that these lower water activities are dehydrating the dissociation transition state preceding dissociation of the protein from DNA. The energies associated with exclusion of solute are quite large at these high pressures.

The comparatively small sensitivity to salt activity seen for the equilibrium between specific and non-specific binding shown in Figure 7 is consistent with other measurements of DNA-binding proteins[4,12,33,34] that also show slightly more ions coupled with non-specific binding than with specific binding. This dependence can be analyzed either as a difference in ion binding reflecting a difference in DNA-protein charge interactions between specific and non-specific complexes or as an indirect, osmotic effect of salt. If analyzed as an ion-binding effect, the data are consistent with a release of an additional 0.6 ion in forming the non-specific complex from the specific sequence complex. This small increase could be due to closer interactions of non-specifically bound protein charge with DNA phosphate groups on the backbone. More likely, however, this dependence is due to the osmotic contribution of salt. As long as salt is excluded from the same water-filled cavities as the neutral solutes, an osmotic effect is required. If salt acts osmotically on the equilibrium between specific non-specific binding with the same $\Delta N_{w,nsp-sp} = -110$ molecules as for the neutral solutes, then the osmotic contribution would be equivalent to the release of ∼ 0.5 ion over the salt concentration range examined. The data are not sufficiently precise to distinguish unambiguously salt binding from an osmotic effect.

As with the osmotic-dependence, the total $\Delta N_{salt}^{\dagger}$ of 5.8 ions at pH 7.5 can be apportioned between an apparent −0.6 ion for $\Delta N_{salt,nsp-sp}$ and +6.4 for $\Delta N_{salt,2}^{\dagger}$, i.e. there is a net binding of six ions linked to the dissociation of non-specifically bound protein. Since the dependence of the association rate on salt concentration shows a net release of about five ions,[4] the transition state between non-specifically bound and fully dissociated protein still has about half the charge interactions of the non-specific or specific complex.

The inversion of salt activity sensitivity of the dissociation rate (Figure 6(a)) at NaCl concen-

trations less than about 50 mM has been observed for the specific site equilibrium binding constant.[14] The enzymatic parameter K_m shows a loss of salt-sensitivity at concentrations less than about 50 mM,[15] but with added Mg^{2+}. Figure 6(b) shows that the change in salt-dependence is also accompanied by a loss of sensitivity to water activity. It has been suggested that the effect of lower salt on the equilibrium is due to protein aggregation.[14] We observed, however, no significant change in the fraction of specific recognition sites initially bound with protein or loss of protein during the dissociation experiment. Additionally, osmotic stress might be expected to exacerbate aggregation rather than have decreased effect. The loss of sensitivity to salt activity suggests that the rate-limiting step now occurs before dissociation of the protein from the DNA fragment. The insensitivity of the dissociation rate to a fourfold change in the concentration of the acceptor DNA fragment indicates the protein is not transferring directly from one DNA fragment to another *via* a collision complex in the rate-limiting step. The decrease in $\Delta N_w^{\dagger}$ indicates that the transition state for this step is less hydrated than the non-specifically bound complex at these lower salt concentrations. The very slow dissociation kinetics precludes measurement of the osmotic-dependence of $K_{nsp\text{-}sp}$ at these lower salt concentrations.

The dissociation rate is strongly dependent on pH in the range 6.5-9.2 (Figure 8), as has been observed for the specific association binding constant.[14] There is little dependence of $\Delta N_w^{\dagger}$, however, on pH (Figure 10). A small decrease ($\sim$15 %) is observed at the high osmotic stresses needed at pH $\sim$8.5. This, once again, may reflect a small dehydration of the transition state associated with dissociation of the non-specifically bound protein or a small, non-linear, solute-linked change in salt binding and activity at the lower water activities. There is no observable effect of pH on the equilibrium between specific and non-specific binding.

Not surprisingly, there is a coupling of salt ion and pH-sensitivities over the pH range 6.5-8.5 (Figure 8). The difference in $\Delta N_{salt}^{\dagger}$ between pH 6.5 and 8.5 is -1.9 ± 0.5. The pH titration curve of $\ln(k_d)$ constructed from overlapping pH regions at different osmotic stresses can be fit reasonably assuming two identical groups on the dimeric enzyme. These curves should be difficult to fit precisely, since changing pH at a fixed buffer concentration will change the ionic strength of the solution and, therefore, indirectly change the dissociation rate through ion binding. Without knowing the relative partitioning of Na^+ and imidazole$^+$ or Tris$^+$ binding to DNA, this affect cannot be corrected straightforwardly. One could additionally expect other protein groups to begin titrating at pH values of 6.5 and 8.5, further complicating the fit of the titration curve. We can only speculate on the identity of the titrating group. His114 makes direct contact with the DNA phosphate backbone in the specific complex. Additionally, His13 is within the disordered N-terminal region not seen in the electron density profile of the co-crystal. This region has been shown to affect strongly both the dissociation rate and the cleavage reaction itself.[16,31,32]

Conclusions

The apparent number of water molecules coupled with the dissociation rate of specifically bound *Eco*RI is seen insensitive to pH between about 6-9 and to salt concentration between about 60-130 mM NaCl. Conversely, the salt and pH-dependence of dissociation is also seen insensitive to osmolyte concentration over a wide range. Water activity acts as a thermodynamic variable that is independent from salt or pH. The change in equilibrium or rate constants with water activity therefore provides valuable additional information about DNA-protein binding thermodynamics. At least for *Eco*RI, the sensitivities to salt concentration, pH, and water activity reflect different aspects of protein binding. If we take the specific complex as a base, then the dissociation reaction in the salt range between 75 and 125 mM NaCl, and using betaine or methyl glucoside to set water activity can be schematically represented as:

$$[\text{Specific complex}] \Leftrightarrow [\text{Nonspecific complex}] \cdot 110\ H_2O \Rightarrow [\text{Transition complex}]^{\dagger} \cdot 125\ H_2O \cdot 6Na^+$$

The dissociation step between non-specific complex and the transition state complex is also pH-sensitive, but the number of bound protons will depend on pH. The number of bound water molecules associated with the transition state complex, but not with the non-specific complex, is dependent on the particular solute used.

The osmotic dependence is primarily due to differences between specific and non-specific binding of the protein. Both salt and pH-sensitivities are mostly due to the non-specific binding of free protein and do not distinguish between specific and non-specific binding. The modest number of water molecules coupled with dissociation of non-specifically bound protein compared with the non-specific-specific equilibrium, the contrasting large contribution of salt and pH to this rate, and the seeming insensitivity of this rate to bulk solution viscosity suggests a discrete protein conformational change is the rate-limiting step in dissociation. The sensitivity of the non-specifically bound protein dissociation rate to the identity of the osmolyte indicates that the conformational change likely entails a change in solute-accessible surface area.

Another thermodynamic parameter that is seen significantly different for specific and non-specific complexes is the change in heat capacity.[1,3,35] Specific binding is generally accompanied by comparatively large changes in heat capacity. In con-

trast, non-specific binding typically results in very little heat capacity change. Since the change in heat capacity is now considered due to the thermodynamics of water hydrating the DNA and protein surfaces and to the consequent release of this water in forming the specific complex, the differences between specific and non-specific complexes in osmotic dependence and heat capacity change are necessarily related.

Finally, osmotic stress offers a practical tool for stabilizing or slowing the dissociation rate of weak or transient complexes for isolation or physical characterization. This approach can be applied to any complex that sequesters water from the bulk solution. Since osmolytes, unlike salts, for example, can in principle be added to very high concentrations (~3 m here for α-methyl glucoside and betaine) without significantly changing structures, very large extents of stabilization can be achieved.

Materials and Methods

Materials

DNA fragments containing a single *Eco*RI recognition site were isolated from the plasmid pNEB193 (New England Biolabs) using standard techniques. The 360 bp fragment was purified from a *Pvu*II (New England Biolabs) digestion of the plasmid and the 240 bp DNA fragment from a *Hin*dIII-*Bgl*I (both New England Biolabs) digestion of the plasmid. The self-complementary oligonucleotide used as a competitor DNA in specific-non-specific competition equilibrium experiments was $(dI\text{-}dC)_{12}$. The oligonucleotide was purchased from Gibco BRL, dissolved in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA), heated to 92 °C, and annealed by slow cooling to 25 °C. Small molecular mass impurities were removed using P6 Bio-Spin columns at room temperature. The double-stranded character of the oligonucleotide was confirmed by polyacrylamide gel electrophoresis. The concentrations of the DNA fragments and oligonucleotide were determined spectrophotometrically, using an extinction coefficient of 0.0148 $(\mu M \text{ base-pairs})^{-1}\ cm^{-1}$ at 250 nm for $(dI\text{-}dC)_{12}$ and 0.013 $(\mu M \text{ base-pairs})^{-1}\ cm^{-1}$ at 260 nm for the DNA fragments. Absorption spectra were obtained with a Shimadzu UV-2101 PC spectrophotometer.

DNA-binding experiments were done mostly with highly purified *Eco*RI restriction endonuclease (kindly provided by Dr L. Jen-Jacobson). To allow comparison with our previous results, equilibrium competition and dissociation kinetics experiments were partly repeated with *Eco*RI purchased from New England Biolabs and used without further purification. Active protein concentrations were determined by direct titration with the 240 bp DNA fragment containing the specific recognition sequence under conditions of stoichiometric binding as described.[5,6]

Betaine glycine was purchased from United States Biochemical, sucrose and glycerol from Mallinckrodt, stachyose and TMAO (trimethylamine N-oxide) from Sigma Chemical Co., α-methyl glucoside, triethylene glycol and t-butanol from Fluka, and DMSO (dimethyl sulfoxide) from Calbiochem. All solutes were used without further purification. Osmolal concentrations of betaine, sucrose, α-methyl glucoside, stachyose, triethylene glycol, TMAO and glycerol were determined by direct measurement using a vapor pressure osmometer operating at room temperature (Wescor, Logan, UT; model 5520XR). Since both DMSO and t-butanol have significant vapor pressures at 20 °C, the osmometer cannot be used for these solutes. Osmolal concentrations for these two solutes therefore were taken as equal to molal concentrations under assumption that at low concentrations (<1 M) these solutes are close to ideal. In comparison to the other solutes, this may result in overestimating ΔN_w by ~10 % at most. Changes in water chemical potentials are linearly proportional to solute osmolal concentrations, i.e. $\Delta\mu_w = \mu_w - \mu_w^{ref} = RT \ \ln(a_w/a_w^{ref}) = -RT$ (osm)/55.6, where μ_w and μ_w^{ref} are the water chemical potentials of the solutions with and without added osmolyte, respectively, and a_w and a_w^{ref} are the water activities of the solutions with and without added osmolyte. Solution viscosities with the various osmolytes were measured using a Gilmont Instruments Falling Ball viscosimeter. Water and glycerol solutions were used to calibrate the system.

Dissociation kinetics

Solution conditions for kinetic experiments were 20 mM imidazole for the pH range 6.2-8.4 or 20 mM Tris-HCl for the range 8.0-9.2. Unlike Tris, pK_a values for imidazole are insensitive to osmolyte identity for a wide variety of solutes. The salt concentration varied between 40 and 160 mM NaCl. All samples contained 2 mM EDTA, 1 mM DTT, 0.1 mg ml^{-1} BSA, and 2.5 % (w/v) Ficoll (70,000 molecular mass) and were incubated at 20 °C. The total reaction volume was 30 µl. In the absence of Mg^{2+}, we observed no measurable cleavage of the DNA.

The experimental protocol we employ is a modification of the standard dissociation rate method. *Eco*RI (~1 nM) was initially incubated with the 240 bp DNA fragment containing the specific *Eco*RI recognition site (~2 nM) under conditions of virtually stoichiometric binding and allowed to incubate at 20 °C for approximately ten minutes. The 360 bp DNA fragment derived from the same plasmid and containing the *Eco*RI recognition sequence was then added. The reaction mixture was allowed to incubate for a fixed time and then electrophoresed to separate *Eco*RI-bound and free DNA fragments.

Equilibrium competition experiments

Equilibrium competition experiments were performed as described.[5,6] Briefly, mixtures of *Eco*RI (~1 nM), the specific site 240 bp fragment (~2 nM), and the oligonucleotide competitor, $(dI\text{-}dC)_{12}$ (between 0 and ~60 µM in oligonucleotide or 0- ~ 1.4 mM in bp), were incubated at 20 °C for 90 minutes. The loss of specific site binding as the concentration of non-specific competitor DNA increases was determined by a gel mobility shift assay. In the experiments reported here, the salt concentration was varied in the range 90-160 mM NaCl at pH 8.0 (20 mM imidazole). Competition experiments were also performed at pH values of 7.0 and 8.0 (20 mM imidazole) with 120 mM NaCl. All samples also contained 2 mM EDTA, 1 mM DTT, 0.1 mg ml^{-1} BSA, and 2.5 % Ficoll. Total reaction volume was 30 µl. Under all these conditions, the specific site dissociation rate is sufficiently fast compared to the 90 minute incubation time to ensure equilibrium. As a control, reactions were also quenched by adding a 50-fold excess (in *Eco*RI recog-

nition site concentration) of a 24 bp oligonucleotide containing the specific binding site and adding the osmolyte triethylene glycol to a final concentration of 2 m to greatly slow the dissociation rate (by a factor of about 300). No difference in competition was observed using the quenching reaction.

Gel mobility-shift experiments

Reaction mixtures from equilibrium and kinetic experiments were electrophoresed in a 10% (w/v) polyacrylamide gel, 0.5 × TAE (45 mM Tris, 22.5 mM acetic acid, 1 mM EDTA, pH 8.3) buffer. Samples were loaded onto a gel at 150 V, and gel was run for 15 minutes. The voltage was then reduced to 46 V and the gel was run overnight at 20 °C to separate free DNA fragments and *Eco*RI-bound complexes. *Eco*RI-specific DNA fragment complexes are remarkably stable in polyacrylamide gels, no change in fractions of bound fragment was observed for 8-16 hours run.

Quantification and data analysis

Electrophoretic bands containing free DNA fragments and DNA-protein complexes were visualized and quantified using the fluorescent dye SYBR Green I (Molecular Probes). The gels were photographed with a Panasonic BD 400 videocamera (averaging 128 frames) using epi-illumination in the UV and a high-pass 495 nm optical filter or with a Luminescent Image Analyzer LAS-1000plus (Fuji Film) that includes a 1.3 megapixel cooled CCD camera, epi-illumination at 470 nm (LED), and an optical filter suitable for SYBR green I. Both cameras were connected to a Macintosh IIfx microcomputer. Band intensities were quantified using NIH Image for Windows (Scion Corp., version Beta3b). The linearity of fluorescent intensity versus DNA amount per band over the range of concentrations studied was confirmed using pBR322 DNA fragments generated by *Msp*I digestion.

Kinetic data analysis

Monitoring both the loss of enzyme binding to the 240 bp fragment and the concomitant increase in binding to the 360 bp fragment allows simplifications in the kinetic analysis. If A and AP represent the free and *Eco*RI bound 240 bp DNA fragment, B and BP the free and *Eco*RI bound 360 bp fragment, and P the free protein, then the simplest reaction scheme is:

$$\begin{aligned} A + P &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} AP \\ B + P &\underset{k_2}{\overset{k_1}{\rightleftharpoons}} BP \end{aligned} \qquad (10)$$

We neglect any length-dependence of k_1 or k_2 between the 240 and 360 bp fragments. Since the concentration of free *Eco*RI, [*P*], is much less than the total protein concentration, P_0, under conditions of virtually stoichiometric binding, then the above equations can be solved with a steady-state approximation for P to give for the fraction, $f(t)$, of fragment A and B with bound protein:

$$\begin{aligned} f_A(t) &= f_0 e^{-k_d t} + f_\infty (1 - e^{-k_d t}) \\ f_B(t) &= f_\infty (1 - e^{-k_d t}) \end{aligned} \qquad (11)$$

The parameter f_0 is the fraction of initially bound 240 bp fragment ($=P_0/A_0$, where A_0 is the total concentration of 240 bp fragment); f_∞ is the final fraction of bound 240 or 360 bp fragment ($=P_0/(A_0 + B_0)$); and the dissociation rate constant $k_d = k_2/(1 - f_\infty)$. Since $A_0 f_A(t) + B_0 f_B(t) \equiv A_0 f_0$, it is convenient to recalculate and plot the data as:

$$\ln(F(t)) = \ln\left(\frac{(f_A(t) - f_B(t))}{(f_A(t) + \frac{B_0}{A_0} f_B(t))}\right) = -k_d t \qquad (12)$$

More complicated reaction schemes are considered in the Discussion.

Equilibrium competition data analysis

As was developed previously,[5,6] the ratio of specific (sp) and non-specific (nsp) association binding constants ($K_{nsp-sp} = K_{sp}/K_{nsp}$) can be determined from the loss of specifically bound complex as the concentration of a non-specific oligonucleotide competitor DNA is increased. If f_b and f_b^0 are the fractions of *Eco*RI-bound specific sequence fragment with and without added oligonucleotide competitor, then under conditions of virtually stoichiometric protein binding and for much weaker non-specific than specific binding ($K_{nsp} \ll K_{sp}$), the change in binding can be represented by:

$$f_b = -\frac{1}{K_{nsp-sp}} \frac{f_b}{1 - f_b} \frac{[DNA_{oligo}]_{total}}{[DNA_{sp}]_{total}} + f_b^0 \qquad (13)$$

The relative binding constants, $K_{nsp\,-\,sp}$, were calculated straightforwardly from the slopes of f_b *versus* $(f_b/1 - f_b)[DNA_{oligo}]_{total}$, measured at constant specific site DNA and protein concentrations. The difference in the numbers of solute-excluding water molecules between specifically and non-specifically bound protein is calculated from the dependence of K_{nsp-sp} on the solute osmolal concentration by:

$$\frac{d \ln(K_{nsp-sp})}{d(osm)} = -\frac{\Delta N_{w,nsp-sp}}{55.6} \qquad (14)$$

where $\Delta N_{w,nsp-sp} = N_{w,sp} - N_{w,nsp}$.

pH titration fits

The experimental pH-dependence of the specific site dissociation rate curve was fit to equations commonly used to describe equilibrium reactions. For a single titrating group, if the dissociation rates of the unprotonated and the protonated forms are k_d^0 and $k_d^0 k_d^*$, respectively, and if the equilibrium constant for protonation of the specific complex is K_h, then the expected dependence of the dissociation rate on proton concentration, $[H^+]$, is:

$$k_d = k_d^0 \frac{(1 + K_h[H^+]k_d^*)}{(1 + K_h[H^+])} \qquad (15)$$

For two identical titrating groups, each of which changes the dissociation rate by a factor equal to k_d^* when protonated, the expression is:

$$k_d = k_d^0 \frac{(1 + K_h[H^+]k_d^*)^2}{(1 + K_h[H^+])^2} \quad (16)$$

The titration data were fit to these equations using the non-linear regression routines of SigmaPlot 5 (SPSS, Inc.).

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